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Reduced Expression of CD20 Antigen as a Characteristic Marker for Chronic Lymphocytic Leukemia

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The surface antigens expressed by the cells of chronic lymphocytic leukemia (CLL) are well known. Most CLL are monoclonal B-cell lymphoproliferative disorders characterized by the coexpression of B-cell antigens and CD5, an antigen present predominantly on T cells. Very little attention, however, has been paid to the quantitative characteristics of the expression of B-cell antigens in CLL. In this study, we used flow cytometry to analyze the expression of CD20, a well-known B-cell-associated antigen, in lymphocytes from 42 cases of CLL and its tissue counterpart, small lymphocytic lymphoma (SLL), and compared the results with results obtained from the analysis of 21 follicular lymphomas, 20 hyperplastic reactive nodes, and 26 samples of normal peripheral blood. The intensity of CD20 expression in the CLL/SLL cells was significantly lower than that of B cells in the other categories. This antigen expression abnormality does not appear to be a universal phenomenon in CLL/SLL, since CD19, another pan-B antigen, was expressed in CLL/SLL at levels higher than those in follicular lymphomas and comparable to those in reactive lymph nodes. These results indicate that the low CD20 expression can be used as a marker for CLL/SLL. The few cases exhibiting intense CD20 expression may represent a biologically different disease. CLL/SLL cells faintly expressing CD20 also show concomitant low CD5 expression in a manner not observed in normal CD5-expressing B cells. © 1992 Wiley-Liss, Inc

Key words: B cells, CLL, flow cytometry, fluorescence, lymphomas

INTRODUCTION

Chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL) are clonal proliferation of small lymphocytes involving peripheral blood, bone marrow, lymph nodes, spleen, and other organs. During the 1980s, major immunological advances and the introduction of monoclonal antibodies led to numerous studies aimed at characterizing the surface antigens expressed by CLL cells [1–4]. The majority of these studies focused on the presence or absence of expression of surface antigens on the leukemic cells, and very few considered the antigenic density as an important biological characteristic of the neoplastic cells [5]. In this study, we analyzed the expression of a variety of cell surface antigens on CLL and SLL cells by flow cytometry and specifically quantitated the intensity of the expression of CD20, a B-cell-associated antigen [6]. The results were compared with those of low-grade follicular lymphomas and of B cells from hyperplastic reactive lymph nodes and normal peripheral blood.

MATERIALS AND METHODS

Samples

Samples were obtained from materials submitted to us for diagnostic purposes. They included 36 peripheral blood specimens from patients with CLL, six lymph node biopsies from patients with SLL, 21 lymph node biopsies

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from patients with low-grade follicular lymphomas (FL), and 20 hyperplastic reactive nodes (RN) submitted to rule out lymphoma. Normal peripheral blood (NPB) specimens were obtained from 26 healthy volunteers (aged 25–65 years). The diagnosis of these samples was based on hematological, morphological, and immunological criteria [7,8]. All cases diagnosed as CLL had increased number of circulating mature-looking lymphocytes, which labeled as B cells coexpressing CD5. These cells expressed either no detectable surface immunoglobulins or a single immunoglobulin light chain.

Tissue Processing

A portion of each lymph node was fixed in 10% buffered formalin and processed for routine histological examination. Single cell suspensions from lymph nodes were prepared as described elsewhere [9]. Peripheral blood mononuclear cells were obtained by centrifugation through a ficoll hypaque gradient. Peripheral smears were prepared and stained by the Wright Giemsa method for routine morphological examination.

Immunofluorescence Staining

Direct immunofluorescence staining with monoclonal or polyclonal antibodies was performed as described previously [9]. Briefly, antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were placed at appropriate dilutions in the wells of microtiter plates [10]. One hundred twenty thousand cells were placed in each of the wells, incubated for 15 mins on ice, and washed in buffered saline solution twice. Antibodies in this study included monoclonal reagents against CD2 (Leu 5B), CD3 (Leu 4), CD4 (Leu 3A), CD5 (Leu 1), CD7 (Leu 9), CD8 (Leu 2A), CD10 (CALLA), CD19 (Leu 12), CD20 (Leu 16), and HLA-DR (HLA-DR) and polyclonal antibodies against individual immunoglobulin light chains. Controls included FITC- and PE-conjugated isotype-matched normal mouse IgG. All monoclonal antibodies were obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA), and the polyclonal antibodies were from either Caltag Laboratories (South San Francisco, CA) or Kallestad Laboratories (Austin, TX). Dual-color (FITC/PE) immunofluorescence combinations included antibodies against CD19/kappa, CD19/lambda, and CD20/CD5. According to the manufacturer, the various lots of antibodies against CD20 used in this study were conjugated with FITC at F/P ratios ranging from 4.7 to 4.9, and the variation in mean fluorescence intensity of normal blood B cells stained with the various lots of reagents did not exceed 4%.

Data Analysis

Data were collected on a Facscan flow cytometer (Becton Dickinson Immunocytometry Systems). The fluorescence data were collected with logarithmic amplification, and Lysys software (Becton Dickinson Immunocytometry Systems) was used to convert the logarithmic data into linear-equivalent fluorescence values. Ten thousand cells were collected in each sample, and data were stored in list mode. Expression of the various antigens was visually determined on forward light scatter/fluorescence intensity bivariate plots, by comparing binding of specific antibody with that of the isotype-matched IgG control. Intensity of CD20 and CD19 expression of lymphocytes was calculated by dividing the peak value of the linear-ized fluorescence of the positive distribution by that of the isotype-matched IgG control. The assessment of immunoglobulin light chain expression was performed by analyzing immunoglobulin light chain distributions of the CD19-expressing cells only.

TABLE I. Intensity of Expression of CD20 on B cells

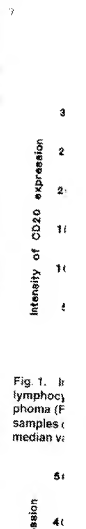
Diagnosis[a] (No. of cases)	CD20 intensity (median)	*P* value[b]	CD19 intensity (median)	*P* value[b]
CLL/SLL (42)	13.7		16.4[c]	
FL (21)	84	<0.0005	10	<0.005
RN (20)	67.9	<0.0005	19	ns
NPB (26)	90.4	<0.0005	ND[d]	—

[a]CLL, chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; FL, low grade follicular lymphoma; RN, reactive nodal hyperplasia; NPB, normal peripheral blood.
[b]Values were calculated by comparing cases from each group to those of CLL/SLL using the Kruskal-Wallis two-sample test. ns, Not significant.
[c]Only 41 cases analyzed.
[d]ND, not done.

RESULTS

In addition to B-cell antigens (CD19 and CD20) and CD5, CLL/SLL cells expressed HLA-DR but lacked expression of T-cell markers such as CD2, CD3, CD4, CD7, and CD8. No surface immunoglobulin was detected in two cases, and the rest showed expression of a single immunoglobulin light chain (28 kappa, 12 lambda), which was often faint. CD10 was not expressed in any of the CLL/SLL cases. All cases of FL demonstrated HLA-DR, CD19, and CD20 but lacked CD5 expression. FL also showed single immunoglobulin light chain expression (15 kappa, 6 lambda), and CD10 was present in 76% of the cases. In contrast, only 15% (3/20) of the RN expressed CD10. None of the RN demonstrated a restricted light chain expression.

The intensity of CD20 expression in the 42 cases of CLL/SLL is shown in Table and Fig. 1. The median fluorescence intensity of CD20 in CLL/SLL was 13.7 (range 1.5–174), whereas the median values in FL, RN, and NPB were 84 (range 12–298.6), 67.9 (range 16–174), and 90.4 (range 40–199), respectively. The differ-



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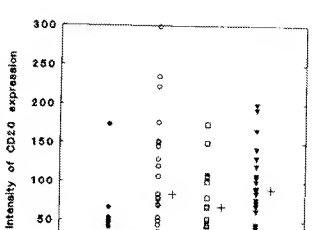

Fig. 1. Intensity of CD20 expression in 42 cases of chronic lymphocytic leukemia (CLL), 21 cases of follicular lymphoma (FL), 20 cases of reactive lymph nodes (RN) and 26 samples of normal peripheral blood (NPB). Crosses indicate median values.

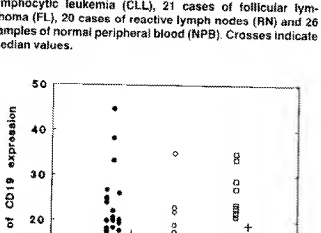

Fig. 2. Intensity of CD19 expression in 41 cases of chronic lymphocytic leukemia (CLL), 21 cases of follicular lymphoma (FL) and 20 cases of reactive lymph nodes (RN). Crosses indicate median values.

ences between CD20 intensity of expression in CLL/SLL and that of each of the other groups were highly statistically significant (Kruskal-Wallis 2-sample test, $P < 0.0005$). The median fluorescence intensity of CD19 was 16.4 (range 6.4–44.8) for CLL/SLL, 10 (range 4–35) for FL, and 19 (range 3.5–34.9) for RN. In this study, CD19 antibodies were not tested in NPB. There was no significant difference between the intensity of CD19 expression in CLL/SLL cells and in B cells of RN. However, CD19 expression in CLL/SLL cells was more intense than in B cells of FL ($P < 0.005$) (Fig. 2).

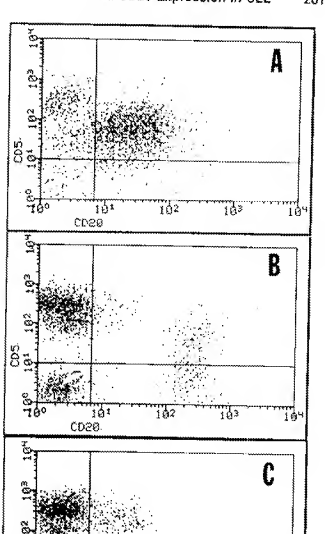

Fig. 3. Dual color analysis of CD20 and CD5 expression in a case of chronic lymphocytic leukemia (A) and normal peripheral blood (B, C). Cells were simultaneously exposed to FITC-labelled anti-CD20 and PE-labelled anti-CD5. The majority of cells in A exhibit faint CD20 and CD5. No cells with similar intensity of expression are seen in B and C. Normal blood lymphocytes coexpressing CD20 and CD5 demonstrate either normal CD20 and faint CD5 expression (B) or normal CD5 and faint CD20 expression (C).

Simultaneous dual-color correlated immunofluorescence analysis was performed in NPB to quantitate CD20 and CD5 coexpressing cells which may represent putative normal counterparts of CLL cells. This analysis revealed the presence of two discrete small subpopulations of lymphocytes coexpressing CD5 and CD20. One of the subpopulations expressed CD20 antigen with a fluores-

cence intensity similar to that of normal B cells but with less CD5 expression than normal T cells (Fig. 3B). The other subpopulation showed exactly the opposite pattern, with a relatively bright CD5 expression (similar to normal T cells) and a relatively dimmer CD20 expression in comparison to the normal B cells (Fig. 3C). Because of their relatively low numbers and the faint expression of either antigen, a precise quantitation of these subpopulations was not possible. In all of our CLL/SL cases, CD5 was expressed at lower levels than in normal T cells (data not shown). Thus, with the exception of those few cases when CD20 was detected in normal amounts, CLL/SL cells did not coexpress CD20 and CD5 antigens in the same manner as NPB lymphocytes.

DISCUSSION

The immunophenotypic analysis of CLL/SLL, FL, RN, and NPB performed in our laboratory confirms in general the data published in the literature [1–4,11,12]. However, our study demonstrates the unique feature of faint intensity of CD20 expression on CLL/SLL cells. CD20 intensity expression on neoplastic B cells has not been thoroughly examined previously. We are aware of four publications in which this analysis was specifically addressed [5,13–15]. Our results are similar to those of Cossman et al. [13], who found that the cells of ten SLL cases had less CD20 expression than either follicular center cell lymphoma or intermediately differentiated lymphocytic lymphomas. Likewise, Marti et al. [5] found a reduced expression of CD20 reagents in five cases of CLL. On the other hand, our findings are somewhat different from those of Freedman et al. [14], who found that CD20 was expressed strongly on CLL cells. We also cannot confirm the high frequency of bright CD20 expression in CLL described by Maddy et al. [15], who divided CLL cases into two approximately equal groups on the basis of the intensity of expression of high- and low-molecular-weight leukocyte common antigen (LCA) and found high expression of CD20, CD21, and CD22 in the subgroup with denser expression of high-molecular-weight LCA. These discrepancies may be due, in part, to different staining procedures, to dissimilar fluorochromes, or to the cell separation procedures used.

One may argue that, although in our study antibodies were used in saturating amounts, CLL samples contain many more B cells than samples of normal blood. Thus the decreased staining with CD20 antibody could be due to insufficient reagent available in the preparations to saturate the B-CLL cells. This is very unlikely, since the low CD20 expression is not observed in other B-cell lymphoproliferative disorders containing the same relative number of B cells, and residual CD5-negative normal B cells sometimes present in CLL/SL samples express normal levels of CD20 (data not shown).

It is important to emphasize that the values of fluorescence intensity reported in this paper apply only to the cases studied in our laboratory and could not be directly compared with results obtained with different reagents and/or other instruments. The results of the measurements could be standardized if they were expressed as antibody binding sites per cell. This would require the use of standard particles to measure quantitatively both the molecules of equivalent fluorochrome and the average fluorescence intensity per antibody molecule for each reagent [16].

In the past, attempts to correlate immunophenotypic analysis with prognosis in CLL were unsuccessful [17–19]. However, it should be noted that these studies dealt primarily with presence or absence of surface antigens, with little emphasis on the intensity of expression. In our series, very few cases of CLL show intense CD20 expression. We cannot determine at this time the clinical relevance of this observation, but the density of surface CD20 may indicate a biologically relevant phenomenon in CLL and may represent a variable of prognostic value. In fact, we frequently observed intense CD20 expression in cases of prolymphocytic leukemia studied in our laboratory (data not shown).

Many observers have correlated phenotypic markers of B-CLL with those expressed at various stages of maturation of normal B cells. Cossman et al. [13] hypothesized that the tumor cells in SLL are more differentiated and closer to plasma cells than those of follicular and intermediately differentiated lymphoma cells. Indeed, the notion that CLL/SLL cells are at a late stage of differentiation is substantiated by in vitro studies showing that activation of B cells leads to a characteristic phenotype resembling that of B-CLL [14]. Although we did not study the maturation sequence of neoplastic B cells, we show that there is a decrease in the intensity of CD20 in B cells of RN compared with NPB, suggesting that stimulation/activation and perhaps further differentiation of B cells may be associated with a decrease in CD20 expression. It is believed that CD20 functions as a calcium channel (T. Tedder, personal communication) and that it regulates a step in B cell activation that is required for further differentiation [20]. This suggests that B cells at different stages of differentiation may express different levels of CD20. However, it is uncertain whether the surface antigens of CLL/SLL cells reflect a unique stage of normal B cell differentiation or represent an abnormal phenotype due to neoplastic transformation.

The few B cells that normally coexpress CD5 and CD20 may expand in autoimmune disorders [21–23] and may be the target for neoplastic transformation in CLL. The dual color analysis performed in our samples in healthy donors confirmed the presence of these cells in small percentages. However, we found two populations of such cells, and, judging by their antigenic density, we

believe that neither of the two coexpresses CD5 and CD20 in a manner comparable to the surface characteristics of the typical CLL/SLL.

In summary, our data indicate that dim CD20 expression is a unique feature of CLL/SLL cells. The small number of CLL/SLL cases in which cells show intense expression of CD20 antigens may represent a biologically or even clinically different disorder. This group will require further study. Immunophenotypically, the CD5-positive B cells present in NPB do not resemble CLL/SLL cells.

Note Added in Proof

After submission of this manuscript, we became aware of a study in press in NY Acad Sci entitled "CD20 and CD5 Expression in B-Chronic Lymphocytic Leukemia (B-CLL)" by G.E. Marti, G. Faguet, P. Bertin, J. Agee, G. Washington, S. Ruiz, P. Carter, V. Zenger, R. Vogt, and P. Noguchi. Using a quantitative approach, these authors also found significantly less expression of CD20 on B-CLL cells than in normal B-cells from peripheral blood. They also confirmed the lower expression of CD5 on the leukemic cells in comparison to normal T-cells.

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